APPENDIX A

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Oligonucleotide-targeted degradation of U1 and U2 snRNAs reveals differential interactions of simian virus 40 pre-mRNAs with snRNPs

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ABSTRACT

We have investigated the roles of U1 and U2 snRNP particles in SV40 pre-mRNA splicing by oligonucleotide-targeted degradation of U1 or U2 snRNAs in *Xenopus laevis* oocytes. Microinjection of oligonucleotides complementary to regions of U1 or U2 RNAs either in the presence or absence of SV40 DNA resulted in specific cleavage of the corresponding snRNA. Unexpectedly, degradation of U1 or U2 snRNA was far more extensive when the oligonucleotide was injected without, or prior to, introduction of viral DNA. In either co-injected or pre-injected oocytes, these oligonucleotides caused a dramatic reduction in the accumulation of spliced SV40 mRNA expressed from the viral late region, and a commensurate increase in unspliced late RNA. When pre-injected, two different U2 specific oligonucleotides also inhibited the formation of both large and small tumor antigen spliced early mRNAs. However, even when, by pre-injection of a U1 5′ end-specific oligonucleotide, greater than 95% degradation of the U1 snRNA 5′ ends occurred in oocytes, no reduction in early pre-mRNA splicing was observed. In contrast, the same U1 5′ end oligonucleotide, when added to HeLa splicing extracts, substantially inhibited the splicing of SV40 early pre-mRNA, indicating that U1 snRNP is not totally dispensable for early splicing. These findings confirm and extend our earlier observations which suggested that different pre-mRNAs vary in their requirements for snRNPs.

INTRODUCTION

Splicing of eukaryotic messenger RNA precursors (pre-mRNA) is a complex process that involves small nuclear ribonucleoprotein particles (snRNPs) (for review see refs. 1–3). These particles each consist of several proteins associated with one or two small nuclear RNAs, termed U snRNAs. The identification of individual snRNPs that participate in splicing in animal cells was accomplished principally by inactivating their function using either antibodies that recognize protein components of the snRNPs or oligonucleotides that are complementary to discrete regions within the various U snRNAs, and which result in degradation of the complementary RNA by RNase H. Such approaches were used successfully in extracts of cultured mammalian cells (4,5), in yeast (6), and in microinjected *Xenopus laevis* oocytes (7,8). In particular, snRNPs containing U1 (4,5), U2 (9,10), U5 (11,12), and U4/U6 (13,14) snRNAs were shown to be required for splicing of mRNA precursors *in vitro*. During the process of pre-mRNA splicing, snRNPs were observed to be stably (U2, U5, and U4/U6) or unstably (U1) associated with the precursor RNA, forming a multicomponent complex, termed the splicesome (see ref. 3). The roles of U1 and U2 snRNPs in splicing have been most extensively investigated. The U1 snRNP binds to the 5′ splice site in pre-mRNA (15), a process which involves base-pairing between the 5′ splice site of the pre-mRNA and the 5′ end of U1 snRNA, both of which are highly conserved sequences (16). The U2 snRNP binds to the branch site region (9), a process

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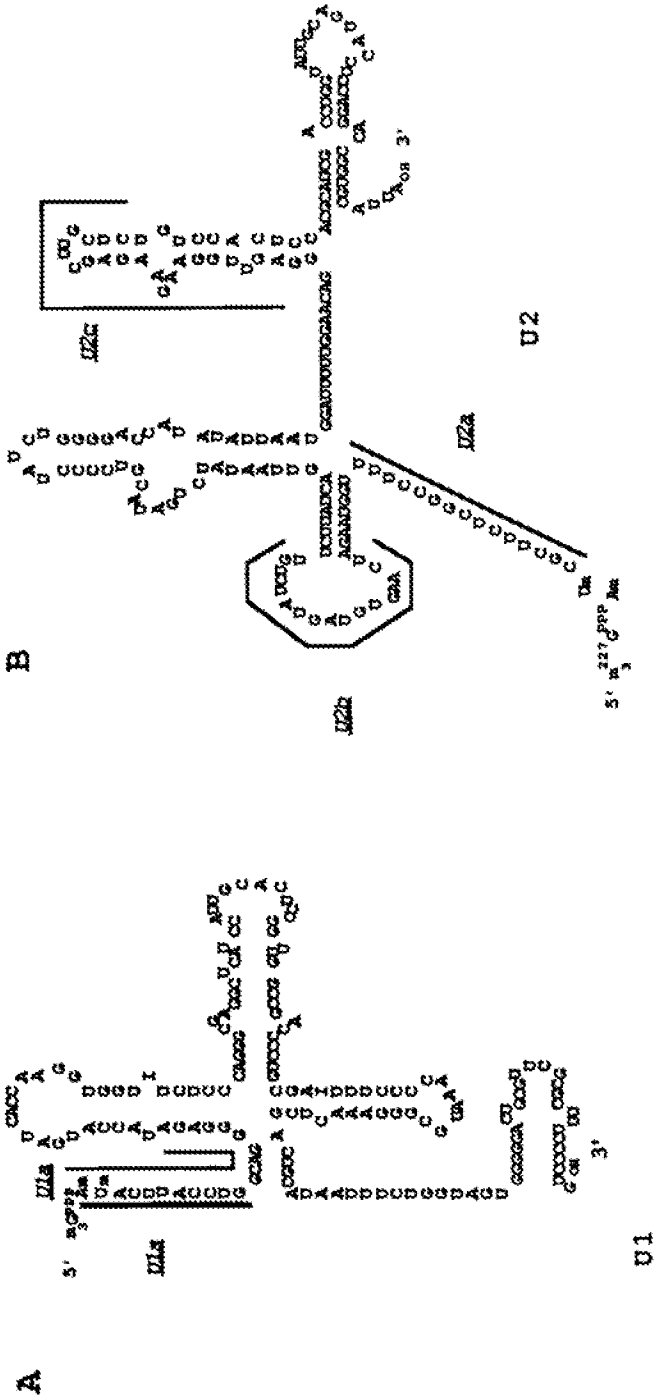
B
U2c
U2a
U2
U2b
A
U1a
U1a
U1

which requires an additional factor, designated U2AF (17). The yeast U2-like snRNA base-pairs with sequences in the branch site (18), and recent evidence suggests that this is also the case in higher eukaryotes (G. Wu and J.L. Manley, and Y. Zhuang and A.M. Weiner, manuscripts submitted).

The majority of studies identifying the roles of individual snRNPs in splicing have utilized cell-free extracts that are capable of splicing precursor RNAs to their spliced products. In order to understand further the roles of these particles in cells, we have utilized *Xenopus laevis* oocytes. The mature oocyte is capable of correctly splicing several pre-mRNAs (7,19,20). It was shown that lariat-intron intermediates are formed (21) and, in some cases, accumulate in oocytes (22), suggesting that the amphibian oocyte shares similar splicing mechanisms with those observed in mammals.

It was shown previously that both SV40 late and early coding regions are expressed after microinjection of viral DNA into the nucleus of *Xenopus laevis* oocytes (23). The viral late pre-mRNA is spliced predominantly into the 19S RNA class, while splicing of early region RNA precursor produces both the large T and the small t antigen mRNAs (7,24). Our previous studies have indicated that both U1 and U2 are necessary for SV40 late pre-mRNA splicing in oocytes. This was first demonstrated by the observation that splicing of late RNA was abolished in oocytes that had been injected with antibodies which exclusively interact with the U1 snRNP (7). We then showed that both U1 and U2 snRNPs were necessary using oligonucleotide targeted degradation (8). Our earlier experiments using anti-RNP and anti-Sm antibodies indicated that the early and late region pre-mRNAs differ in their interaction with snRNPs (7). While injection of all anti-Sm or anti-U1 (RNP) antibodies tested abolished splicing of SV40 late pre-mRNA, some of these antisera did not block early pre-mRNA splicing, while others inhibited the formation of large T but not small t antigen spliced mRNAs. In the present study we have extended these observations by examining the roles of the U1 and U2 snRNPs in splicing of the early and late SV40 pre-mRNAs, using oligonucleotide-targeted degradation.

MATERIALS AND METHODS

Oligonucleotides

All oligonucleotides used in this study were chemically synthesized on an Applied Biosystem DNA synthesizer, and purified by sephadex G-50 chromatography. The sequences for U1a, U2b, and C oligonucleotide were as previously described (6). The other oligonucleotides were: U1s CAGGTAAGTA, U2a AAAGGCCGAGAAGCG, and U2c GAGCAAGCTCTTCTTCCAAC.

Preparation of Precursor RNA

Capped precursor RNA containing SV40 early region (nt 5171–4002) was prepared by *in vitro* transcription with SP6 RNA polymerase as previously described by Noble et al (19).

Microinjection of X. laevis Oocytes

Oocytes were prepared and microinjected using methods described previously (7). For DNA injection, SV40 DNA form I was injected intranuclearly in quantities of 2.5 ng per oocyte. Oligonucleotides were injected into oocyte nuclei in nanogram quantities, as indicated in each experiment. For RNA injection, 1 ng of capped pre-mRNA was injected

Figure 1. *Oligonucleotides complementary to regions within U1 or U2 snRNA.* The predicted structure for oocyte U1 snRNA is from Forbes et al (35), for U2 is from Mattaj and Zeller (45). The oligonucleotides complementary to regions of U1 or U2 snRNA are indicated by solid lines.

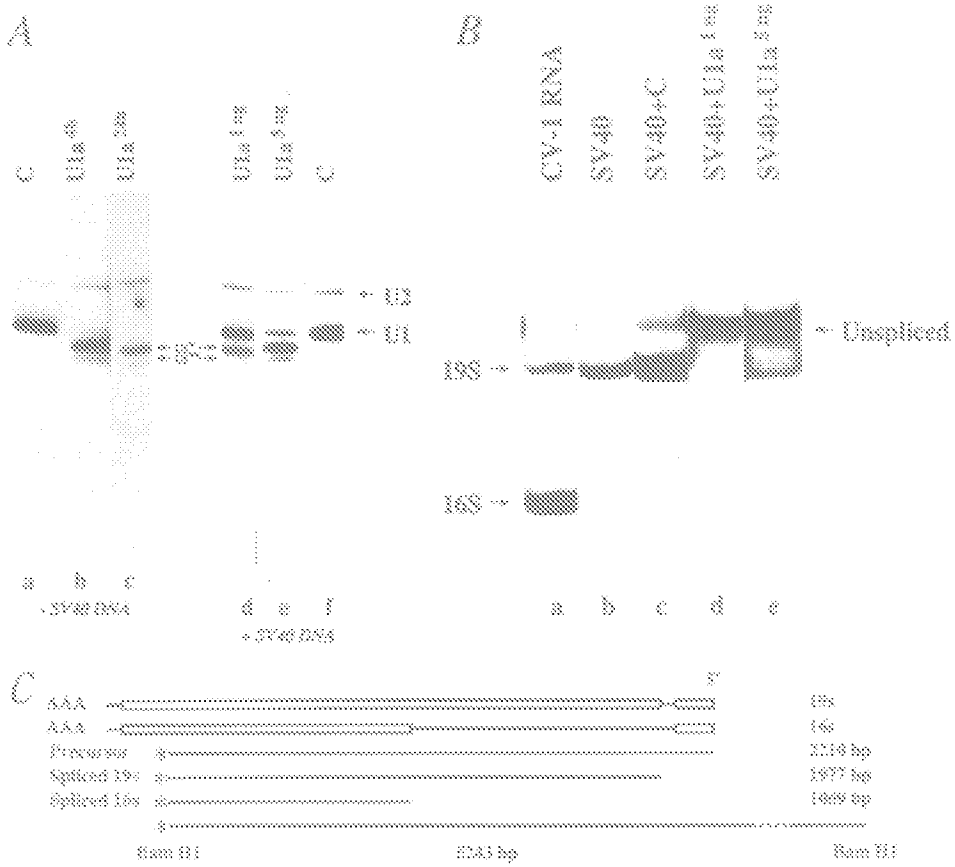

Figure 2. *Degradation of U1 snRNA 5' ends blocks SV40 late pre-mRNA splicing in oocytes.* (**A**) Northern analyses of U1 snRNA in oocytes. Left panel: Groups of ten oocytes were injected intranuclearly with 20 ng of C oligonucleotide (lane a) or 5 ng of U1a (lanes b and c). The injected oocytes were incubated for 24 hours (lanes a and c) or 4 hours (lane b), and RNA was extracted and analyzed. Right panel: groups of 10 oocytes were co-injected with 2.5 ng of SV40 DNA and either 1 ng of U1a (lane d), 5 ng of U1a (lane e), or 20 ng of C oligonucleotide (lane f). (**B**) SV40 late RNA splicing is blocked in oocytes injected with U1a. Groups of ten oocytes were injected with 2.5 ng of SV40 DNA (lane b–e) in the presence of no oligonucleotide (lane b), 20 ng of C oligonucleotide (lane c), 1 ng of U1a (lane d), or 5 ng of U1a (lane e). After 24 hrs, RNA was extracted and subjected to lane a represents cytoplasmic RNA from CV-1 cells 48 hours after SV40 infection that had also been subjected to S1 nuclease analysis. (**C**) The diagram indicates the sizes of DNA fragments generated by S1 analysis from unspliced and spliced late RNAs.

into the nucleus of an oocyte. Generally, oocytes were injected in groups of ten, followed by incubation in modified Barth's solution (25) at 19°C for 16–24 hours unless otherwise specified in the text.

RNA Analysis

RNA from microinjected oocytes was extracted from pooled oocytes according to the procedures described by Fradin et al (7). Northern analysis was performed using methods

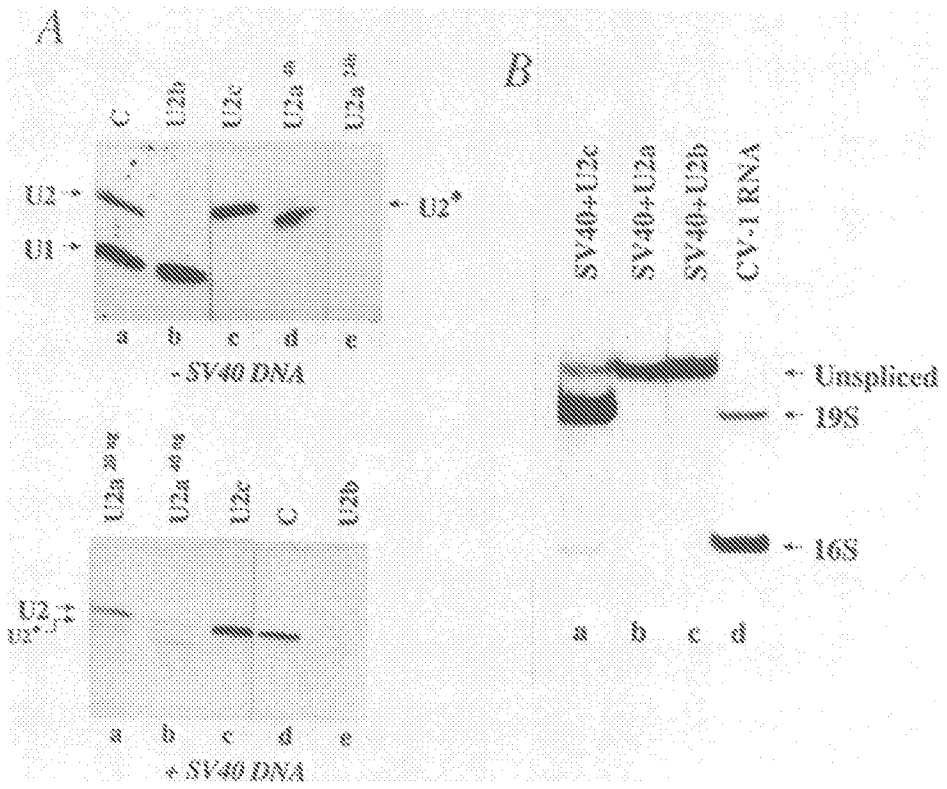

Figure 3. *Degradation of U2 snRNA blocks SV40 late pre-mRNA splicing in oocytes injected with U2-specific oligonucleotides.* **(A)** Northern analyses of U2 snRNA in oocytes. Top panel: oocytes in group of ten were injected with 20 ng C oligonucleotide (lane a), U2b (lane b), U2c (lane c), or U2a (lanes d and e). The injected oocytes were then incubated for 24 hours (lanes a–c, e), or 4 hours (lane d) before extraction. Bottom panel: oocytes were injected with 2.5 ng of SV40 DNA along with 20 ng of U2a (lane a), U2c (lane c), C oligonucleotide (lane d), or U2b (lane e), or with 40 ng of U2a (lane b). RNA extracted from oocytes was analyzed by Northern blotting. **(B)** Degradation of U2 snRNA inhibits splicing of SV40 late RNA in oocytes. Groups of ten oocytes were injected with 2.5 ng of SV40 DNA along with 20 ng of U2c (lane a), U2a (lane b), or U2b (lane c). S1 nuclease analysis was performed on RNA samples from one oocyte equivalent.

described previously (8). In all cases, quantities of extracted RNA equivalent to one oocyte were used. For S1 nuclease mapping of spliced SV40 late RNAs, a labelled DNA probe was prepared and hybridized to extracted RNA equivalent to one oocyte according to conditions described by Michaeli and Prives (26). The products from S1 mapping were analyzed on 1.2% denaturing glyoxal-agarose gels. For analysis of spliced viral early RNA, S1 mapping was performed following procedures described by Fradin et al (7) using RNA from one oocyte equivalent for hybridization. After purification, the S1-resistant fragments were subjected to 5% polyacrylamide-urea electrophoresis (27).

In Vitro Splicing

In vitro splicing using HeLa cell extracts was carried out essentially as described by Noble et al (21). Treatment of HeLa splicing extracts with oligonucleotides and RNase H was performed using conditions established by Black et al (9).

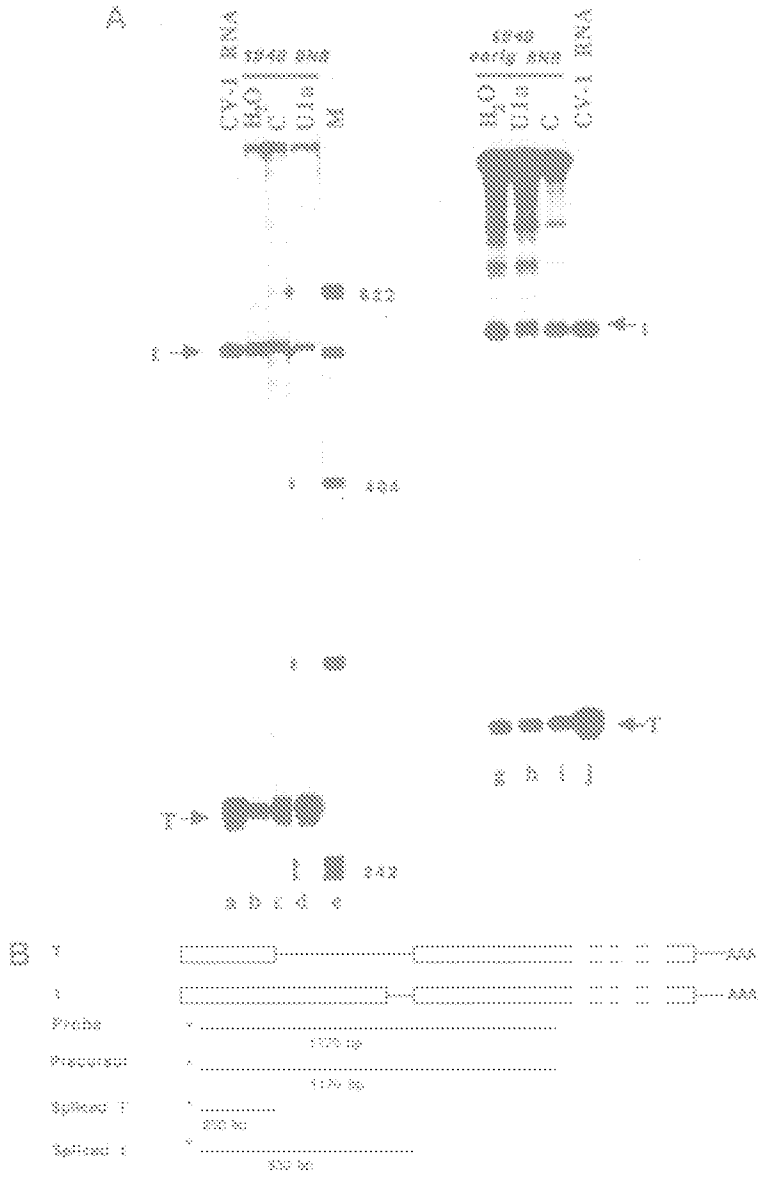

Figure 4. *U1a does not block SV40 early splicing oocytes.* (**A**) S1 nuclease analysis of early RNAs. Left panel: 2.5 ng of SV40 DNA was injected into oocytes, in groups of ten, along with H_2O (lane b), 20 ng of C oligonucleotide (lane c), or 5 ng of U1a (lane d). Lane a, RNA from infected CV-1 cells. Lane e contains labeled pBR322 DNA fragments. Right panel: 4 hours after injection with H_2O (lane g), 5 ng U1a (lane h), or 20 ng C oligonucleotide (lane i), oocytes received a second injection of SV40 early precursor mRNA that was synthesized *in vitro*. RNA extracted from these injected oocytes, in amounts equivalent to one oocyte, was subjected to S1 analysis, using a 3′ end labeled single stranded DNA probe. (**B**) The diagram indicates the S1 nuclease resistant DNA fragments corresponding to spliced and unspliced early RNAs.

RESULTS

Partial Degradation of U1 or U2 snRNA Inhibits SV40 Late pre-mRNA Splicing

The five oligonulceotides complementary to either U1 or U2 snRNA are diagrammed in Figure 1. Oligonucleotides U1a (20 mer), U1s (10 mer) and U2a (20 mer) are complementary to the 5′ ends of U1 or U2 snRNAs, while U2b (15 mer) and U2c (38 mer) are complementary to the loop and stem region of U2 snRNA, respectively. Oligonucleotides U1a, U1s, U2a, and U2b were chosen because they were reported to block splicing in cell-free extracts (5,9,10). Oligonucleotide U2c was tested initially because it is complementary to a region of U2 snRNA that has homology to the 3′ splice site of SV40 early mRNA (7). Oligonucleotides C and TK are control oligonucleotides containing 15 (5′-TCCGGTACCACGACG-3′) and 30 (complementary to nt 264−293 from the HSV TK gene) nucleotides, respectively, neither of which have homology to any known snRNA sequence.

To study the roles of U1 and U2 snRNPs in splicing in oocytes, these oligonucleotides were injected into oocyte nuclei. The U1a oligonucleotide was introduced in quantities from 80 to 400-fold molar excesses over the estimated concentration of U1 snRNA in oocytes (28) in the presence or absence of SV40 DNA (Figure 2a). RNA was extracted from oocytes 4 or 24 hours after injection of oligonucleotides and subjected to Northern blot analysis using plasmids containing either human U1 or U2 DNA as probes (29,30). Four hours after injection of 5 ng of the U1a oligonucleotide in the absence of SV40 DNA, over 95% of the U1 snRNA was cleaved into smaller products, including a major species (U1*) lacking approximately 7 nt, and a minor species (U1**) resulting from cleavage of about 11 nt (Fig.2A, left panel). These products could still be detected, albeit in smaller quantities, 24 hours after injection of U1a into oocytes. This is most likely related to the stability of the U1 snRNP particle, since the 5′ truncated U1 snRNA can still be recovered from anti-Sm immunoprecipitates after this time period (8,9). By contrast, co-injection of U1 into oocytes along with SV40 DNA resulted in relatively less degradation of U1 snRNA, 1 ng and 5 ng induced cleavage of approximately 40% and 70% of U1 5′ ends, respectively (Fig.2A, right panel). Although not well understood, speculations on these differences can be made (see Discussion).

To test the effects of the U1a oligonucleotide on SV40 late pre-mRNA splicing, SV40 DNA was injected alone, or along with either the control C oligonucleotide, or with the U1a oligonucleotide (Figure 2B). RNA was extracted from oocytes 24 hours after injection and subjected to S1 nuclease mapping as previously described (26) using 5′ end-labeled SV40 DNA cut with Bam H1 as a probe. This allowed us to map 3′ splice site utilization. As previously published (7,24), oocytes splice SV40 late pre-mRNA efficiently, but the utilization of the available splice sites is markedly different from those observed in infected monkey cells. Thus while the major late RNA in infected cells is the 16S class, the major oocyte spliced SV40 late RNA is the 19S class. Injection of the C oligonucleotide had no effect on the appearance of spliced 19S RNA in oocytes, whereas injection of 5 ng of U1a drastically reduced accumulation of spliced 19S RNA, and increased the quantities of unspliced viral late RNA (Fig. 2B). Furthermore, as little as 1 ng of U1a inhibited SV40 late RNA splicing to the same extent as 5 ng of this oligonucleotide. As 1ng destroyed only 40% of the U1 snRNA 5′ ends, it is likely that formation of the SV40 late 19S splice in Xenopus laevis oocytes depends upon the presence of the majority of the endogenous U1 snRNPs.

The effects of oligonucleotides complementary to the 5′ end or internal regions of U2

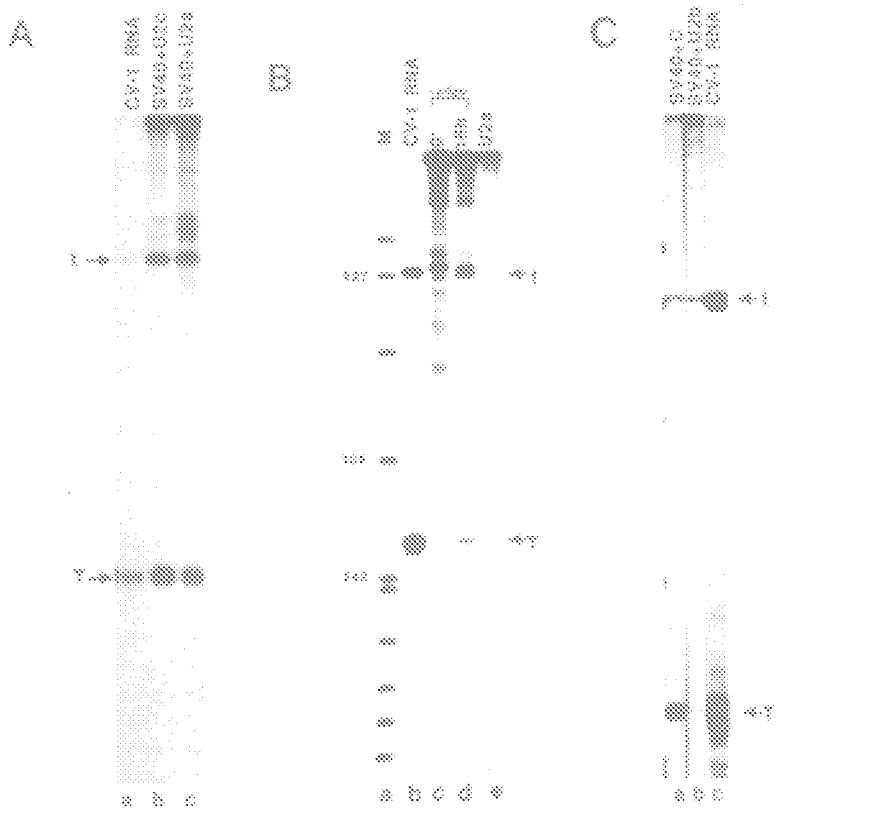

Figure 5. *Intact U2 snRNA is required for splicing of SV40 early pre-mRNAs in oocytes.* (**A**) Oocytes were injected with 2.5 ng of SV40 DNA along with 20 ng of U2c (lane b) or U2a (lane c). After 24 hrs, RNA was extracted and analyzed by S1 nuclease protection as in Figure 4. (**B**) *In vitro* synthesized SV40-1 early precursor RNA was injected into oocytes along with H_2O (lanes c and d) or 20ng of U2a oligonucleotide (lane e). RNA was extracted from oocytes immediately (lane c) or 16h (lanes d and e) after injection and analyzed as in A. (**C**) Splicing of SV40 early pre-mRNA in oocytes injected with U2b. Groups of ten oocytes were injected with 2.5 ng of SV40 DNA in the presence of 20 ng of C oligonucleotide (lane b), or U2b (lane c). RNA extracted 24 hours after injection was analyzed as in A.

snRNA were similarly tested (Figure 3). In contrast to what was observed with the U1 oligonucleotide, 5—10 fold greater quantities (2000 to 4000-fold molar excesses over the estimated quantity of U2 snRNA; I. Mattaj, personal communication) of the U2-specific oligonucleotides than of the U1-specific oligonucleotide were required in order to bring about U2 snRNA degradation. The reasons for this are not clear, as approximately similar quantities of U1 or U2 snRNPs are present in oocytes. U2 snRNA was analyzed by Northern blotting 4 or 24 hours after injection into oocytes (Figure 3A). As before, the C oligonucleotide did not affect the integrity of U2 snRNA. Injection of U2b resulted in the disappearance of any detectable U2 snRNA, while injection of U2a resulted in a U2 snRNA species in which over 90% was truncated. However, this shorter U2 snRNA disappeared within 24 hours incubation (compare lanes d and e, top panel), suggesting that degradation of the 5′ ends of U2 snRNA eventually destabilized the entire molecule.

This is in contrast to U1 snRNA, in which we observed that the 5′ end-cleaved snRNA was stable for at least for 24 hours (Fig. 2A, left panel, lane c). U2c had no effect on the endogenous U2 snRNA, most likely because the homologous region of U2 snRNA was not available to form a hybrid with the oligonucleotide. As had been observed with U1 degradation, co-injection of U2 oligonucleotides with SV40 DNA led to reduced degradation of U2 snRNA (Fig. 3A, bottom panel). In order to cleave 70% of U2 snRNA 5′ ends, 40 ng U2a oligonucleotide was required. This is based on the observed overall reduction in the quantity of detectable U2 RNA, and is consistent with our observation that the stability of U2 snRNA appeared to be more susceptible to 5′ end degradation than that of U1 snRNA in oocytes. However, with or without co-injected SV40 DNA, the U2b oligonucleotide caused the disappearance of any detectable U2 snRNA within 24 hours. S1 nuclease analysis using the same method as described above of SV40 late mRNA in these oocytes showed that U2c had no effect on splicing of the viral late pre-mRNA, consistent with its inability to induce U2 snRNA degradation. Both U2a and U2b abolished the appearance of spliced 19S RNA. Thus, degradation of the majority of the oocyte endogenous U2 snRNA abolished splicing of SV40 late mRNA in oocytes.

Cleavage of the 5′ End of U1 snRNA Does Not Inhibit Splicing of SV40 Early pre-mRNA in Oocytes

It was observed previously that SV40 early region transcripts are synthesized and spliced in oocytes to give rise to both large T and small t mRNAs (5). It was also shown that, while injection of either anti-Sm or anti-RNP antibodies inhibited late pre-mRNA splicing, early RNA splicing was less affected. The formation of small t mRNA was not blocked by any antibodies tested in oocytes. These results suggested that early and late viral mRNAs exhibit different interactions with snRNPs, in particular the U1 snRNP. However, these antibody experiments did not provide the sensitivity and specificity obtainable with oligonucleotide targeting. Therefore, to extend these previous experiments, we analyzed SV40 early splicing following treatment with the oligonucleotides described above. S1 analysis as previously described, using single-stranded end-labeled DNA probe, allowed us to analyze the effects of partial degradation of U1 snRNA on SV40 mRNA splicing (Figure 4). As can be seen in lane b of Fig. 4A, the ratio of t to T mRNA was altered in oocytes when compared to that in infected monkey cells, confirming previous observations (7). Surprisingly, injection of the C oligonucleotide resulted in the synthesis of relatively more large T than small t spliced mRNA. This effect was also observed with the TK oligonucleotide (data not shown), indicating that injection of unrelated oligonucleotides can affect the splicing of SV40 early pre-mRNA. We (8) and others (31) have observed that oligonucleotides are unstable in oocytes such that within 2 hours after their injection they are degraded virtually completely to nucleotides. The effect on the ratios of spliced mRNAs therefore most likely did not depend upon the presence of intact oligonucleotides.

Co-injection of the U1a oligonucleotide along with SV40 DNA, in concentrations that abolished late pre-mRNA splicing within the same batch of oocytes, failed to inhibit either large T or small t splicing. Because we had observed that injection of oligonucleotides in the absence of SV40 DNA induced much more extensive cleavage of U1 and U2 snRNAs, U1a was preinjected into oocyte nuclei 4 hours before a second injection with SV40 DNA. However, even when by pre-injection of the U1a oligonucleotide greater than 95% of the endogenous U1 snRNA was cleaved (e.g. as in Fig 2A), there was still no significant effect on splicing of SV40 early pre-mRNA in oocytes (data not shown).

It was previously shown that microinjection of capped SV40 early precursor RNA

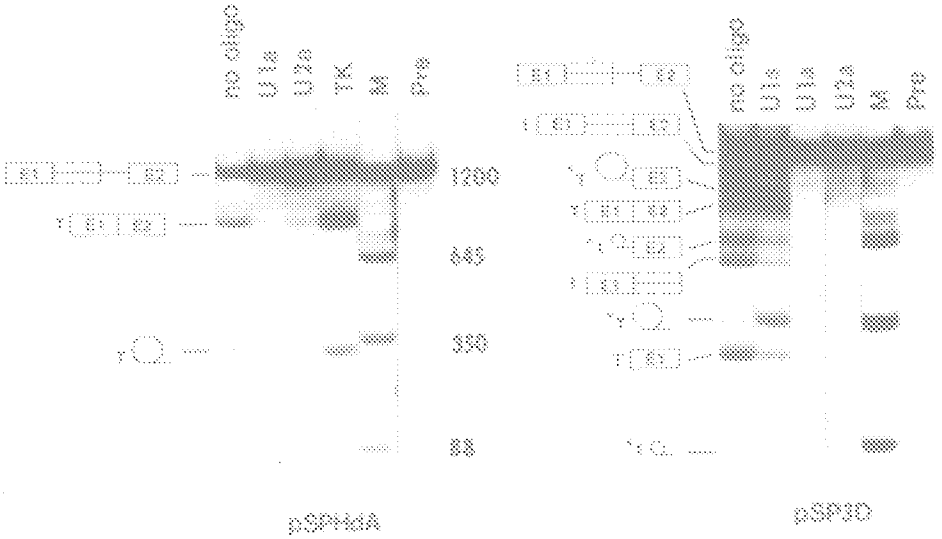

Figure 6. *Splicing of SV40 early substrates in oligonucleotide-treated HeLa extracts.* HeLa nuclear extract was preincubated with the indicated oligonucleotide and RNase H, and then incubated with ^{32}P-labeled wild-type SV40 early transcript (pSPHdA) or 3' splice site duplication mutant transcript (pSP3D). Extracted RNAs were then analyzed on denaturing 1.4% agarose gels. The major splicing products are diagrammed at the left of each gel. Sizes in nt indicated at the right; Pre: unincubated precursor. Splicing products from the duplication mutant are indicated by an asterisk (see text).

synthesized *in vitro* leads to formation of both spliced T and t mRNA in oocytes (20). We were therefore able to determine whether the U1a oligonucleotide affected splicing of the viral early RNA precursor in the absence of ongoing transcription, by injecting the *in vitro* transcript into oocytes that had been previously injected either with water, U1a or C oligonucleotide (Fig 4A, right panel). Under all conditions, both T and t mRNA were produced with almost equal efficiency. Note that in this case the relative abundance of large T to small t spliced RNA was not affected by the presence of oligonucleotides. Therefore, the effect of the injected oligonucleotides on T/t mRNA ratio is somehow related to transcription of viral early RNA in oocytes. Although the basis for this phenomenon is not understood, our results confirm that U1 snRNA interacts differently with SV40 early and late pre-mRNAs in oocytes.

U2-Complementary Oligonucleotides Have Varied Inhibitory Effects on Splicing of SV40 Early pre-mRNA in Oocytes

Degradation of oocyte U2 snRNA with the oligonucleotides described above allowed us to examine the role of the U2 snRNP in splicing of SV40 early pre-mRNA. S1 analysis, using the early region-specific probe as described in the previous section, was performed on RNA samples from oocytes coinjected with U2 oligonucleotides and SV40 DNA. As expected from the data shown above, injection of the U2c oligonucleotide did not inhibit SV40 early pre-mRNA splicing, although it also induced the formation of relatively more large T than small t mRNA, as we had observed with the C and TK control oligonucleotides described above (Figure 5A). Co-injection of U2a along with SV40 DNA caused a slight reduction in the quantity of spliced T mRNA, but not t mRNA. However, when either

SV40 DNA (data not shown) or capped precursor mRNA (Figure 5B) were injected into oocytes that had been *previously* targeted with the U2a oligonucleotide, splicing of SV40 early pre-mRNA to both T or t mRNA was inhibited. This finding indicates that U2 snRNA is required for the splicing of SV40 early pre-mRNA in oocytes. Confirmation of this result was obtained from experiments in which preinjection of the U2b oligonucleotide, which resulted in complete disappearance of oocyte U2 snRNA, prior to injection of SV40 DNA, blocked splicing of SV40 early pre-mRNA to both large T and small t mRNAs (data not shown).

As we had shown that in oocytes co-injected with U2b and SV40 DNA, the endogenous U2 snRNA was completely degraded within 24 hours, we expected that both spliced T and t mRNA would also be absent in these oocytes. Surprisingly, however, we observed that while formation of spliced T mRNA that accumulated was inhibited, the quantity of spliced t mRNA was unaffected (Fig. 5C). We have observed that oligonucleotides are virtually completely degraded within the first two hours after injection (8), suggesting that they must target the snRNAs within that time. It is possible that, as SV40 transcripts are produced within that time frame (32), the presumably small quantities of intact U2 snRNPs 'protected' by the presence of SV40 transcripts might be sufficient to mediate splicing of SV40 early pre-mRNA to t but not T mRNA. Consistent with this is our previously published observation that injection of antisera from some, but not all, systemic lupus erythematosis patients into oocytes inhibited the formation of spliced T but not t mRNA (7). Thus, our data suggest that splicing of the viral early pre-mRNA to t mRNA requires lesser quantities of U2 snRNPs than does its splicing to T mRNA.

SV40 Early pre-mRNA Splicing Exhibits Different Requirements for the U1 snRNP in Oocytes and in HeLa Splicing Extracts

Our results have confirmed and extended earlier experiments in which anti-Sm and anti-RNP antibodies, injected into oocytes, resulted in blocking SV40 late RNA splicing but either did not affect any early RNA splicing or only inhibited large T mRNA splicing. Both the previous and present results suggest that the late and early transcripts have markedly different quantitative requirements for U1 and U2 snRNPs. In fact, the current experiments imply that the U1 RNA 5′ end may not be required for splicing of SV40 RNA in oocytes. Consistent with this possibility, Black and Steitz (13) showed that an oligomer containing 12 nucleotides homologous to the 5′ end of U1 did not block splicing of the SV40 large T antigen mRNA in HeLa cell-free splicing extracts. However, as the oligonucleotide used in our experiments was 8 nucleotides larger than the one used by Black et al., we asked whether our 20-mer might affect the splicing of SV40 pre-mRNA *in vitro*. We previously reported that wild-type SV40 early precursor RNA is spliced almost exclusively to form large T mRNA *in vitro* (27). In contrast, if RNA transcribed from a mutant construct containing a duplication of the 3′ splice site common to both large and small tumor antigen mRNAs (33) is used as substrate in HeLa splicing reactions, then among the RNA products are those that have undergone cleavage and splicing of the small t 5′ splice site and the duplicated 3′ splice site (unpublished results; see below). Wild-type or the 3′ splice site duplication mutant precursor RNAs were added to HeLa nuclear extracts that had been pretreated with either U1a, U2a, U1s (complementary to the 5′ 10 nucleotides of U1 snRNA) or the control TK oligonucleotides and the RNA products were resolved by polyacrylamide gel electrophoresis. Both U1a and U2a treated extracts were virtually unable to splice either wild-type or duplicated pre-mRNAs. The results shown in Figure 6 indicate that U1 snRNA had been degraded. However, treatment with the U1s oligonucleotide had

little or no effect on large T splicing using the mutant precursor RNA and blocked the accumulation of small t related splicing intermediates by approximately 50%. In all cases, analysis of the endogenous snRNAs revealed that greater than 80% of the appropriate U snRNA had been degraded (data not shown). Thus, in contrast to the result obtained in oocytes, the U1a oligonucleotide was able to block splicing of SV40 early pre-mRNA *in vitro*. That the U1s oligonucleotide in our experiments or the U1 12-mer used by Black et al. failed to block early splicing suggests that a more extensive region of the 5′ end of U1 snRNA may be required for early pre-mRNA splicing. Our results therefore show that conditions can be established demonstrating a role for the U1 snRNA 5′ end in SV40 RNA splicing. They suggest, however, that there are different requirements for U1 snRNA for early mRNA splicing in oocytes and in cell free extracts.

DISCUSSION

Injection of oligonucleotides into oocytes has proven useful for targeted degradation of mRNA (see ref. 34 for review) and of snRNAs (8). By using oligonucleotide-induced degradation of sequences within U1 and U2 snRNAs, we have been able to examine the fate of the snRNAs targeted and the ensuing effect upon the splicing of both early and late SV40 pre-mRNA transcripts in *Xenopus laevis* oocytes. Several points can be made from our experiments. (1) While both 5′ ends of U1 and U2 snRNA are susceptible to cleavage after injection of the appropriate oligonucleotide into oocytes, significantly more snRNA cleavage occurred when oligonucleotides were injected alone as opposed to when they were co-injected with SV40 DNA. (2) After 5′ end degradation, U1 snRNA remained stable for at least 24 hours, while U2 snRNA was rendered undetectable within that time. (3) Even when less than 40% of U1 or 70% of U2 snRNA 5′ ends were degraded, late SV40 RNA splicing was almost completely blocked. (4) Early RNA splicing in oocytes exhibited dramatically reduced requirements for both U1 and U2 snRNAs. (5) The effects of the U1 5′ end oligonucleotide on early pre-mRNA splicing differed in *Xenopus laevis* oocytes and HeLa cell-free splicing extracts.

Our experiments showed that when SV40 DNA was co-injected with the oligonucleotides, considerably less U1 or U2 snRNA was degraded. The reasons for this are not entirely clear. The most likely explanation, however, is that snRNAs may be protected if assembled into splicesomes that form as a result of the transcription of 'spliceable' RNA. We have shown that the oligonucleotides are approximately 50% degraded within 30 minutes and are almost completely converted to nucleotides within 2 hours (8). Therefore their effects upon snRNA must be complete within that time frame. It has also been established that after injection of SV40 DNA, there is a lag of approximately 1–2 hours before detectable levels of early and late transcripts are observed, presumably due to organization of DNA into 'active' minichromosomes (35,36). It is therefore possible that a small quantity of snRNPs become inaccessible to oligonucleotides by being assembled into splicesomes associated with the earliest transcripts. There is very little RNA polymerase II transcription of endogenous genes in oocytes (37). Therefore the introduction of the SV40 template may contribute significantly to the quantity of splicing substrates and thus of a source of protection of snRNAs from oligonucleotides. Those snRNPs that have thus escaped the oligonucleotide-arrest would presumably be capable of mediating at least some of the ongoing splicing occurring as transcripts continue to be produced. This notion is supported by our observation that intron-less pBR322 DNA, which is actively transcribed in oocytes (32) failed to protect U1 and U2 snRNA from injected oligonucleotides (unpublished results).

Our experiments also revealed differences in the relative stability of U1 and U2 snRNAs after injection of oligonucleotides. U1 snRNA that had been cleaved at its 5' end by the U1a oligonucleotide remained stable for at least 24 hours, while U2 snRNA, after 5' end degradation by U2a, disappeared within that time. Furthermore, the U2 loop-specific oligonucleotide, U2b, induced complete degradation of U2 snRNA, consistent with previously published experiments (9) using the same oligonucleotide in HeLa splicing extracts. It is likely, in fact, that the U2b oligonucleotide was even more effective in inducing rapid degradation of U2 snRNA than was the U2a oligonucleotide. This is suggested by the fact that even when co-injected with SV40 DNA, in contrast to the U2a oligonucleotide, U2b led to complete disappearance of U2 snRNA. Furthermore, co-injection of the U2a oligonucleotide and SV40 DNA resulted in only a partial reduction in the amount of spliced large T mRNA, while coinjection of U2b completely blocked large T mRNA splicing. Our results therefore suggest significant differences in the stability of the U1 and U2 snRNPs after oligonucleotide-targeted degradation. It should be mentioned that several oligonucleotides complementary to suspected single-stranded regions of U1 snRNA were injected singly or in groups and all failed to block early viral RNA splicing (unpublished data). Therefore it is likely that the accessibility of RNA in the U1 particle is different from that in the U2 particle and this may be related to the relatively greater stability of the former.

We have rather unexpectedly observed that even when as much as 60% of the U1 snRNA remained intact, there was nonetheless a complete block of late splicing in oocytes. It was initially reported by Kramer et al (5) that cleavage of 50% of the U1 snRNA abolished splicing of an adenovirus late pre RNA *in vitro*. However, Krainer and Maniatis (10) later showed that complete inhibition of splicing of a human β-globin pre RNA *in vitro* requires over 90% degradation of U1 snRNA. Our observation that cleavage of 40% of U1 snRNA, or 70% of U2 snRNA, led to almost complete inhibition of splicing of SV40 late pre-mRNA in oocytes seems to be consistent with the former study, but are also consistent with the idea that different pre RNAs display different requirements for snRNPs. It is well established that SV40 viral late RNA is synthesized in large quantities in oocytes (7,23,26). Factors required for splicing these RNA precursors such as the U1 and U2 snRNPs, however, may be limiting. One of our preliminary experiments in which we showed that splicing efficiency can be increased by lowering the quantities of injected pre-mRNAs supports this notion (unpublished results). Thus, most, if not all, of the U1 and U2 snRNPs may be required for splicing of the viral late pre-mRNA. Forbes et al have identified at least 7 species of U1 snRNA in mature oocytes (38). It is not clear whether they play different roles. An unlikely, but possible, explanation of our result is that certain species of U1 snRNA involved in splicing are preferentially cleaved by the oligonucleotide-targeted-degradation. Furthermore, Fakan *et al* have conducted electron microscopic studies suggesting that only a small proportion of snRNPs are located at the actual site of RNA processing (39). It is not clear at present whether the remaining majority of snRNPs are functional in splicing. Thus, it is possible that not all U1 or U2 snRNPs are capable of mediating splicing. Since the oocyte U1 snRNA can be cleaved by oligonucleotide-targeted degradation in a concentration-dependent fashion (this study), we are currently examining the function of the remaining intact U1 snRNPs by introducing *in vitro* synthesized pre-mRNA into these U1a-pretargeted oocytes.

We have shown here that splicing of SV40 early pre-mRNA is insensitive to degradation of over 90% of U1 snRNA in oocytes. Consistent with the *in vitro* observation reported

by Black and Steitz (9), the major cleaved U1 snRNA species after injection of U1a oligonucleotide (20 mer) is approximately 7 nucleotides shorter than the intact U1 snRNA. It was suggested that this truncated U1 snRNA may base-pair with AGGUA sequence spanning the T 5′ splice site (9). However, this argument perhaps can not fully account for this unusual insensitivity. It has been shown that the utilization of the 3′ splice site of SV40 late RNA at nt 558. (see ref. 40) is abolished after treatment with the identical anti-U1 oligonucleotide (6, this study), even though the sequence spanning the 5′ splice site is AGGUU.

There are at least two unusual characteristics in sequences of SV40 early pre-mRNA that interact with U1 and U2 snRNPs. The 5′ splice site of t almost perfectly complements the 5′ end of U1 snRNA (41), and multiple branch sites are used for splicing of T mRNA (21). Either or both of these features may play a role(s) in more efficiently recruiting U1 and U2 snRNPs, thereby quantitatively lowering the requirement for these snRNP particles. We are currently testing the effects of the U1a oligonucleotide on splicing of mutants either lacking the 5′ splice site of t or those eliminating all but one branch site.

Our observation that destruction of the great majority of the U1 snRNA 5′ ends did not affect early RNA splicing in oocytes suggests the possibility that U1 snRNP, or at least the RNA 5′ end, may have no role in the splicing of some pre-mRNAs. However, the ability of the U1a oligonucleotide to block splicing of the early pre-RNA *in vitro* clearly shows that a requirement for the U1 snRNP for splicing of this precursor can be demonstrated in at least one case. Why then, were different results obtained with the oocytes and the cell-free extracts? *Xenopus laevis* oocytes and human HeLa cells are of course very different types of cells. Thus, although commonalities exist between splicing processes in virtually all eukaryotic cells, clear differences in types of U snRNPs, particularly in U1 snRNAs, in amphibian oocytes have been observed (34,35). It therefore remains possible that one of the different types of U1 snRNA detected in *Xenopus* oocytes, which do not exist in HeLa cells, may have either a uniquely high affinity for the SV40 early region in oocytes, a unique resistance to the U1a oligonucleotide, or both. An alternative possibility is that early pre-mRNA is capable of undergoing splicing in a U1-independent fashion in oocytes, but not in HeLa cells. This could be the result of hypothetical factors present within oocytes that obviate the U1 snRNA role in some cases. As the splicing of some group II mitochondrial introns, a process involving 5′ splice site cleavage and lariat formation, does not require any snRNP particles (42.43), and as the U1 snRNP appears to be missing in trypanosomes where trans-splicing of SL RNA actively occurs (44), precedents exist for organelle or organism-specific variations in snRNP requirements. A final possibility is that the degradation of U1 snRNA achieved by the U1a oligonucleotide in HeLa extracts resulted in sufficient destruction of U1 snRNPs so that the concentration of functional particles fell below a 'threshold' that was not reached in oocytes. Although the fraction of U1 RNA degraded *in vitro* was not higher than that detected in the oocytes (unpublished data), it may be that a smaller fraction of the HeLa snRNPs was functional to start with. While this model does not require there be qualitative differences in splicing requirements in HeLa cells and *X. laevis* oocytes, it does support the view that splicing of different pre RNAs can display different quantative requirements for snRNPs. Whatever the reason for the differences we have observed in these experiments, our studies show that not only do different splice sites vary in their interaction with the same set of snRNPs

in a given cell, but that the same splice site, under different conditions, can exhibit differential requirements for the same snRNP. Experiments are in progress to further understand the basis for these differences.

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